Human PEM as a Target for Birth Control and Treatment of Alzheimer's Disease

Description

The invention relates to the human PEM polypeptide, which plays an important role for the maturation of sperm and the nucleic acid that codes for them. The invention comprises the use of PEM as a target in male birth control and for the treatment and diagnosis of male infertility and Alzheimer's disease. The invention also includes a selection process for PEM antagonists as well as the production of binding molecules, which specifically detect PEM. In addition, genes that are regulated by the PEM gene are part of this invention.

The intention to use proteins of the male reproductive tract or sperm proteins as a target group for non-hormonal contraception has been known for several decades. For example, a project with the name "Vaccines for Fertility Regulation" was supported by the World Health Organization (WHO) (P. D. Griffin, Hum. Reprod., 1991, 6: 166-172). Various sperm proteins such as, e.g., PH-20, SP-10, FA-1, FA-2, CS-1, NZ-1, NZ-2 and lactate-dehydrogenase C4 were proposed as candidates for immunocontraception (R. K. Naz, Immunol. Rev., 1999, 171: 193-202). Immunization tests with PH-20 showed that both male and female animals are thus completely and reversibly infertile (P. Primakoff et al., Nature, 1988,

335: 543-546). The use of the intra-acrosomal sperm protein SP-10 as an antigen caused an immunological response in women that reduces fertility (R. W. Wright et al., Biol. Reprod., 1990, 42: 693-701). Active immunization of animals with FA-1 produces a lasting and reversible inhibition of fertility (R. K. Naz and X. Zhu, Biol. Reprod., 1998, 59: 1095-1100).

PEM is a transcription factor that includes the Homeobox family. The corresponding cDNA was cloned from the mouse (M. F. Wilkinson et al., Dev. Biol., 1990, 141: 451-455) and from the rat (S. Maiti et al., J. Biol. Chem., 1996, 271: 17536-17546). PEM transcripts are expressed abundantly and selectively in the male genital tract. In the mouse, the PEM expression was mainly detected in the testes, while in the rat, PEM can mainly be found in the epididymis (K. A. Sutton et al., J. Androl., 1998, 19: 21-30). The in vivo expression of the PEM gene is regulated in these organs by androgens. In addition, PEM transcripts were described in the muscle and in macrophages, but in these cases, the PEM expression does not seem to be regulated by androgens, which can be attributed to the use of different promoters (S. Maiti et al., J. Biol. Chem., 1996, 271: 17536-17546). Despite the unremarkable phenotype of the PEM-knock-out mouse (J. L. Pitman et al., Dev. Biol., 1998, 202: 196-214), it can be assumed that the human PEM plays an essential role in spermatogenesis and/or in sperm maturation. PEM is the sole known transcription factor

whose expression is regulated by androgens (S. Maiti et al., J. Biol. Chem., 1996, 271: 17536-17546).

No one has yet found the human PEM ortholog; this suggests a low sequence conservation in different organisms, as can already be determined by the weak identity (73%) between mouse PEM and rat PEM (S. Maiti et al., Genomics, 1996, 34: 304-316).

The invention relates to the identification of human PEM. Both the complete coding PEM-cDNA sequence and the structure of the PEM gene could be determined. The human PEM-amino acid sequence has only 30% identity with the sequence from the mouse and only 32% identity with the sequence from the rat. The human genomic locus could be defined in Xq 25-26.

The identified cDNA sequence is shown in SEQ ID No. 1, and the protein-coding sequence is shown in SEQ ID No. 2. The genomic sequence could also be identified and is shown in SEQ ID No. 3 (corresponding to a cross-section of nucleotides 16000-170967 from Gene Bank Accession No. AC005023). The initial exon extends from nucleotide 168 439 to 168 042. An internal exon extends from nucleotide 165 491 to 165 446, and the terminal exon extends from nucleotide 161 927 to 161 817 (111 nucleotides). In the range of nucleotides 161 698 to 161 693, there is a polyadenylating signal.

The human PEM is preferably coded by (a) the coding area of the nucleic acid sequence shown in SEQ ID No. 1, (b) one of the sequences according to (a) against the backdrop of the

degeneracy of the genetic code and/or (c) one of the nucleic acid sequences that hybridize under stringent conditions with the sequences according to (a) and/or (b). The human PEM especially preferably has the amino acid sequence shown in SEQ ID No. 2 or an amino acid sequence that is at least 80%, preferably at least 90%, identical to it.

The term "stringent hybridization" according to this invention is used in this case as in Sambrook et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, Laboratory Press (1989), 1.101-1.104). Accordingly, we speak of hybridization under stringent conditions, if after washing for one hour with 1 x SSC and 0.1% SDS at 55°C, preferably at 62°C and especially preferably at 68°C, especially for one hour with 0.2 X SSC and 0.1% SDS at 55°C, preferably at 62°C and especially preferably at 68°C, a positive hybridization signal is still observed. A sequence that hybridizes under such washing conditions with a nucleotide sequence that is shown in SEQ ID No. 1 or a nucleotide sequence that thus corresponds against the backdrop of the degeneration of the genetic code is detected by this invention.

In particular, this invention detects natural, allelic variations of PEM, in which these are optionally also functional mutations. Moreover, recombinant variants, for example functional partial fragments (such as, for example, the "Divergent Paired Class" homeodomains as described for the

mouse of Rayle (Develop. Biol. 146 (1991), 255-257)), are also detected by this invention.

Especially preferably, the human PEM has the amino acid sequence that is shown in SEQ ID No. 2 or a sequence that is at least 80%, and especially at least 90%, identical to it. The 1% identity is in this case calculated according to the following formula:

$$I = n/L \times 100\%,$$

whereby n stands for the number of identical amino acids of the two sequences that are compared to one another and L stands for the length of the sequence section used for comparison.

The inhibition of PEM can result in the inhibition of sperm development or maturation and thus represents a novel approach for contraceptive preparations. In addition, the screening for functional mutations in the PEM gene can be used as a diagnostic agent for determining the causes of infertility. By restoring PEM function (e.g., by gene therapy), patient fertility can also be restored.

The subject of the invention is thus the use of human PEM and/or a nucleic acid that codes for this as a target substance for the production of an agent for birth control.

An inhibition of human PEM can be used for inhibiting fertility and especially for inhibiting spermatogenesis in male mammals. This is of great importance in human contraception, but also in veterinary medicine for population

control. The inhibition of PEM can be carried out by expression reduction by means of antisense-nucleic acids or ribozymes or on the protein level by using inhibitors such as anti-PEM-antibodies or low-molecular antagonists. The production of antisense molecules and ribozymes can be carried out, for example, as described in Sczakiel (Antisense Nucleic Acid Drug Dev. 7 (1997), 439-444, Lavrovsky et al. (Biochem. Mol. Med. 62 (1997), 11-22) and Thompson (Methods Enzymol. 306 (1999), 241-260). Polyclonal antibodies against human PEM can be carried out by immunization of test animals with human PEM or fragments thereof, optionally on a vehicle such as keyhole-limpet-hemocyanin and recovery of the resulting antibodies from the immunized test animal. Monoclonal antibodies can be obtained by, for example, fusion of spleen cells of the immunized test animal with myeloma cells according to the method of Köhler and Milstein or further developments thereof. Low-molecular inhibitors of PEM can be identified by a screening process as explained in more detail below.

By contrast, an activation of human PEM to increase fertility can be used. Also here, applications both in human medicine and in veterinary medicine are possible. The activation of PEM can be carried out by, for example, increasing the PEM expression in target cells, e.g., Sertoli cells in the testes and/or epithelial cells in the epididymis by means of gene-therapy methods. To this end, a nucleic acid that codes for PEM can be introduced into the target cell

under the control of an active promoter in the target cell by means of suitable gene transfer vectors, e.g., viral vectors such as, for example, adenoviruses, retroviruses, adeno-associated viruses or vaccinia viruses, or plasmids, and can be expressed there. Suitable gene therapy processes are described in, e.g., Gomez-Navarro et al. (Eur. J. Cancer, 35 (1999), 867-885). In addition, an activation of PEM can be carried out by low-molecular active substances, which can be identified by a screening process as described below.

Another subject of the invention is a process for the preparation of new agents for birth control. The identification of these new agents is carried out in that the ability of test substances to modulate human PEM is determined. This determination can be performed as a high throughput test, in which a considerable number of test substances is studied in parallel. The test can be performed on a cellular basis, whereby cells can be used that are transfixed with the gene for the human PEM and are able to produce an over-expression of this gene. In contrast, cells can also be tested that contain a completely or partially defective PEM, for example cells that contain a defective human PEM gene in at least one allele, preferably in both alleles. The test cells that are used for the identification of new active substances are preferably mammal cells, especially human cells. As an alternative, a test on a molecular basis can be performed, whereby the human PEM is

used in the form of cell extracts or in an essentially isolated and purified form, optionally also in the form of an active fragment.

In addition, the process according to the invention for identifying new agents for birth control can comprise the formulation of test substances that exert a modulatory action on human PEM, or compounds derived therefrom, into a pharmaceutical agent.

Still another subject of the invention is a diagnostic process, in which the expression and/or the functionality of human PEM is determined in a sample. The sample preferably originates from a patient who is to be subjected to a fertility determination or by a patient in whom the suspicion of Alzheimer's disease exists. The determination of PEM can be carried out on the nucleic-acid level, e.g., on the DNA level, for example by Southern Blot or determination of single nucleotide polymorphisms, on the transcript level by determination of the degree of expression, the expression pattern or the transcript length, or on the protein level, e.g., by immunohistochemical or immunocytochemical methods or by function measurements. The determination of single-nucleotide-polymorphisms allows the identification and diagnosis of functional mutations, which may be the cause of infertility in patients.

In addition to the role in sperm maturation, the human PEM also has a function in the case of Alzheimer's disease.

Human PEM is expressed to an elevated extent in the brain of Alzheimer patients in comparison to brains of healthy humans. The inhibition of PEM can have a positive effect on the course of Alzheimer's disease and thus represents a new starting point for treating this disease. A subject of the invention is therefore the use of human PEM or a nucleic acid that codes for this as a target substance for the production of an agent for treating Alzheimer's disease. The inhibition of PEM can be carried out by a reduction of the expression of PEM. This can be carried out by antisense-nucleic acids, ribozymes or by substances that engage in the regulation mechanism of the PEM-gene expression. Such substances can be identified by a test system that measures the PEM-gene expression. Thus, e.g., cells that are transfixed with the PEM DNA can be brought into contact with the substances to be tested, and the expression of the PEM protein can be demonstrated, e.g., with the aid of antibodies.

In addition, PEM can also be inhibited on the protein level, e.g., by antibodies, peptides or low-molecular antagonists. Since PEM is a transcription factor, it is possible to inhibit the binding of PEM to DNA or the interaction with the transcription machinery.

The subject of the invention is also a process for the preparation of new agents for treating Alzheimer's disease. The identification of these new agents is carried out in that the ability of test substances to inhibit human PEM is

determined. The function of the PEM as a transcription factor is measured. The binding of PEM to DNA can be measured. In contrast, cells can also be used that are transfected (transformed) with the gene for the human PEM. In these cells, the PEM protein is responsible for the gene regulation of other genes, so-called target genes. An inhibition of PEM by the test substances results in a reduction of the expression of the target genes.

Another subject of the invention is a cell that is transfected (transformed) with a DNA that codes for the human PEM or a fragment thereof and that contains at least one exogenous copy of this DNA. Still another subject of the invention is a cell that contains a defective PEM gene in at least one allele, for example a PEM gene that is disrupted by homologous recombination. These cells can be used just like the nucleic acids, which code for human PEM or a fragment thereof, or the human PEM protein itself or a fragment thereof for identifying and characterizing agents for birth control and for treatment of Alzheimer's disease.

Finally, the invention relates to a process for identifying genes that are regulated by the human PEM gene, whereby the influence of human PEM on the gene expression in human cells is tested. This test can be carried out, for example, by transcriptome analysis, e.g., according to the methods described by Kozian and Kirschbaum (Trends Biotechnol. 17 (1999), 73-78) or by proteome analysis according to the

methods described by Dutt and Lee (Curr. Opin. Biotechnol. 11 (2000), 176-179). The genes that are identified by the process and their use as target substance for the production of an agent for birth control or for treatment of Alzheimer's disease are also subjects of this invention.

The invention is explained in more detail by the following figure and example.

Description of the Figure

Fig. 1 shows the expression of human PEM mRNA in tissue samples from various parts of the brain.

A. A sense primer as well as an antisense primer against the protein-coding section of the human PEM RNA were used for the PCR amplification. As a template, first-strand cDNA from various brain parts was used. The PCR products were separated on a 1.5% agarose gel and then stained with ethidium bromide. The human PEM amplificate can be detected at a size of about 550 bp. As size markers, the 1-kb markers and the 100-bp markers of Clontech were used. 1: 100-bp marker; 2: healthy brain, sample-a; 3: healthy temporal lobe; 4: temporal lobe with Alzheimer's disease; 5: healthy frontal lobe; 6: frontal lobe with Alzheimer's disease; 7: healthy hypocampus; 8: hypocampus tumor; 9: fetal brain;

10: healthy brain, sample-b; 11: water test; 12: 100-bp marker; 13: 1-kb marker.

B. A sense primer and an antisense primer against the protein-coding section of the beta-actin-RNA were used for the PCR amplification. As a template, the same first-strand cDNA as under A. was used. 1: 100-bp marker; 2: 1-kb marker; 3: healthy brain, sample-a; 4: healthy hypocampus; 5: hypocampus tumor; 6: healthy temporal lobe; 7: healthy frontal lobe; 8: temporal lobe with Alzheimer's disease; 9: frontal lobe with Alzheimer's disease; 10: fetal brain; 11: healthy brain, sample-b; 12: water test.

Example

The expression of the human PEM in various brain tissues was analyzed by semi-quantitative PCR. To this end, the following primers were used:

Sense 5'-ATGGCGCGTTCGCTCGTCCACGAC-3',

Antisense 5'-TAGTCCACGACGATGTAGACACAG-3'.

In the control, specific primers for beta-actin were used:

Sense primer 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3',

Antisense primer 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'.

The cDNA was acquired by Invitrogen (Carlsbad, CA, USA). The PCR analysis was performed with the Advantage-2 PCR kit (Clontech). The reaction conditions were as follows: initially 5 minutes at 95°C; then 30 cycles with 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 1 minute; finally 72°C for 7 minutes. The results show an elevated expression of human PEM in the frontal and temporal lobes in Alzheimer patients in comparison to healthy tissue.